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METABOLOMICS CHARACTERIZATION OF U.S. AND JAPANESE F-15 AND C-130 FLIGHT LINE CREWS EXPOSED TO JET FUEL VOLATILE ORGANIC COMPOUNDS AND AEROSOLS

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14. ABSTRACT
Air and ground crews transfer a significant amount of jet fuel, and as a result of transfers, breathe its volatile emission from residues. Working on the flight line also exposes maintainers to exhaust from the jet fuel as engines are tested or run before and after flight. Since little is known concerning level of exposure and the corresponding biological response associated with human jet fuel exposure, nuclear magnetic resonance (NMR)-based metabolomics analysis of human urine was utilized for characterization of metabolite profiles of flight line personnel for potential biomarker discovery. This project was a collaborative research effort between the US Air Force (USAF) and the Japan Air Self-Defense Force (JASDF) to correlate flight line exposure to jet fuel volatile organic compounds (VOC)/exhaust with NMR-derived urinary metabolite profiles obtained from USAF personnel (JP-8 fueled aircraft) and Japanese personnel (JP-4 fueled aircraft) working F-15 and C-130 flight lines. Urine was collected from volunteers at USAF and JASDF air bases located in Japan preshift, postshift and the following morning. Metabolomics data suggested that urinary metabolite profiling may be a useful tool for monitoring flight line personnel exposures to hazardous chemicals. Incorporating select metadata (i.e. total VOC exposure, time on flight line, etc.) influenced NMR spectra to enhance the discriminatory power and accuracy of the metabolomics data analysis. Additional work is still required to identify key metabolites that are predictive of exposure to jet fuels or combustion products.

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Metabolomics, Nuclear Magnetic Resonance Spectroscopy, Jet Fuel, Human, Biomarkers

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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PREFACE

This study was phase two of a cooperative research project conducted under a Memorandum of Understanding between the Department of Defense of the United States of America and the Ministry of Defense of Japan. This international agreement, "The Human Effects of Exposure to Aviation Jet Fuels, JP-4 and JP-8, and Their Engine Exhaust," is a scientific collaboration between the Molecular Bioeffects Branch (711 HPW/RHDJ) and Japan Air Self-Defense Force, Aeromedical Laboratory (JASDF/AML).

The program managers for the Memorandum of Understanding are Asao Kobayashi, PhD for JASDF/AML and David Mattie, PhD for 711 HPW/RHDJ. Funding for this project was equally provided by JASDF and USAF.

The study protocol, Human Operational Exposure to JP-4 and JP-8 Fuel (Exhaust), was approved as FWR20110047H by the Air Force Research Laboratory Institutional Review Board and as 22-01-01 by the Aeromedical Laboratory Ethical Committee

This research was conducted by the Bioeffects Division, Molecular Bioeffects Branch (711 HPW/RHDJ), Human Effectiveness Directorate of the 711th Human Performance Wing of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This technical report was written as the Final Report for 711 HPW/RHDJ Work Unit 7757DH05.

Research performed with Dr. Nicholas Reo, Wright State University, was under Henry M. Jackson Foundation sub-award HJF 132633, contract FA8650-05-2.

The authors would like to acknowledge the men and women of JASF and the USAF that served as subjects for this study. The authors would also like to acknowledge the many JASDF and USAF personnel who assisted in the completion of this study.

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1.0 SUMMARY

Air and ground crews transfer a significant amount of jet fuel, and as a result of transfers, breathe its volatile emission from residues. Working on the flight line also exposes maintainers to exhaust from the jet fuel as engines are tested or run before and after flight. Since little is known concerning level of exposure and the corresponding biological response associated with human jet fuel exposure, nuclear magnetic resonance (NMR)-based metabolomics analysis of human urine was utilized for characterization of excreted metabolite profiles for flight line personnel as a method for potential exposure biomarker discovery. This project was a collaborative research effort between the US Air Force (USAF) and the Japan Air Self-Defense Force (JASDF) to correlate flight line exposure to jet fuel volatile organic compounds (VOC)/exhaust with NMR-derived urinary metabolite profiles obtained from USAF personnel (JP-8 fueled aircraft) and Japanese personnel (JP-4 fueled aircraft) working F-15 and C-130 flight lines. Urine was collected from volunteers at USAF and JASDF air bases located in Japan at preshift, postshift and the following morning time points. The JASDF measured jet fuel chemical components in blood and urine, while the USAF analyzed for potential urinary metabolite biomarkers using NMR-based metabolomics. Blood and urine were collected from volunteers at USAF and JASDF air bases located in Japan. The USAF air bases were Yokota, a C-130 air base and Kadena, an F-15 Air base. The JASDF bases were Komaki, a C-130 air base and Naha and Komatsu, F-15 air bases. In addition, subjects were recruited from Tachikawa air base because there was no active runway at this base and it served as a control air base site to compare with air bases with active runways and significant potential exposure to all on-base personnel. This report describes the methods and results for examining urinary metabolite profiles utilizing NMR-based metabolomics.

In the present study, proton NMR spectra of urine obtained from human subjects were acquired at 25 °C on a Varian INOVA NMR instrument operating at 600 MHz. Metabolomics analysis resulted in the identification of metabolite patterns indicative of flight line exposure when compared to non-flight line control subjects. Regardless of fuel (JP-4 or JP-8) it was found that subjects working with F-15 aircraft received a greater flight line exposure than those subjects who worked with C-130 aircraft. For JP-8, distinct metabolite profiles were observed relative to controls for both F-15 and C-130 workers. Supervised Orthogonal Projection onto Latent Structures - Discriminant Analysis (OPLS-DA) confirmed that flight line subjects working with F-15s, regardless of jet fuel (JP-4 or JP-8), could be classified separately from control subjects with greater discriminating power and statistical confidence than C-130 flight line workers. Furthermore, it was also found that incorporating select metadata into the NMR spectral data analysis enhanced the discriminatory power and accuracy of the metabolomics analysis. Additional work is still required to identify key metabolites (biomarkers) that are predictive of exposure to specific jet fuels or their combustion products. Taken together, our results suggested

that urinary metabolite profiling may be useful as a screening tool for monitoring flight line personnel exposed to hazardous volatile chemicals.

2.0 INTRODUCTION

Due to its wide spread use, JP-8 has been recognized as the single largest source of chemical exposure for U.S. and NATO military personnel (Carlton and Smith, 2000) where inhalation and dermal systems have been shown to represent the primary routes of exposure (Chao et al., 2005). This has resulted in the potential for widespread occupational exposure among military and civilian personnel that may result in central systemic toxicity including the immune system, nervous system and respiratory tract (NRC, 2003). Therefore, a tremendous need exists to identify biomarkers predictive of toxic insult due to exposure of volatile organic chemicals. Previous studies have indicated that urinary naphthalene and its metabolites 1- and 2-naphthol may be effective surrogate markers of exposure to JP-8 (Egeghy et al. 2003; Serdar et al. 2003; Serdar et al. 2004; Chao et al. 2006; Kim et al. 2007). Metabolomics analysis offers a virtually non-invasive sample collection, minimal sample processing, robust and stable analytical platform, with excellent analytical and biological reproducibility that has the potential to identify biomarkers predictive of hazardous flight line exposures.

Metabolomics is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living organisms to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999). This term is derived from the Greek roots "meta" (change) and "nomos" (regularity and order); referring to the ability of chemometric models to classify changes in metabolism (Lindon et al., 2004a). This biotechnology was pioneered by Jeremy Nicholson, Elaine Holmes, and John Lindon in the late "90s" at the Imperial College in London (Nicholson et al., 1999). The field of metabolomics is concerned with the study of fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing (Lindon et al., 2003).

Because metabolites are downstream of both gene transcription and enzyme activities, metabolomics has the potential to give a more accurate picture of the actual physiological state of a cell. Nuclear magnetic resonance (NMR)-based metabolomics has shown great promise as a valuable tool for discovery of the metabolic response to chemical and physical stressors and tissue injury. NMR metabolic analysis using biofluids (i.e. blood, urine, saliva, etc.) is well documented, and the principles of this approach have been described in detail (Robertson, 2000; Lindon et al., 2004b; Lenz et al., 2003; Reo, 2002; Holmes et al., 2000; Holmes and Shockor, 2000; Holmes et al., 1998). Metabolic profiling analysis is performed by finding differences in metabolites across all key parameters.

Spectroscopic methods such as NMR and LC/MS generate enormous complex data sets that are information-rich and necessitate the need for multivariate statistical analysis methods for data interpretation. These analytical techniques provide spectral patterns that can be evaluated using a combination of chemometric and bioinformatic tools to classify biochemical insults that are reflective of specific physiological or pathological states and to identify potential biomarkers resulting from these insults. Evaluation and understanding of these observed biochemical changes over time will provide critical information on mechanisms of action that will lead to the development of novel diagnostic assays and therapeutic treatments for toxicity and disease. An article by Don Robertson (2005) provides an excellent review of the use of metabolomics in toxicology.

In the present study, proton NMR spectra of urine obtained from human subjects were acquired at 25 °C on a Varian INOVA NMR instrument operating at 600 MHz. Metabolomics analysis resulted in the identification of metabolite patterns indicative of flight line exposure when compared to non-flight line control subjects. Regardless of fuel (JP-4 or JP-8) it was found that subjects working with F-15 aircraft received a greater flight line exposure than those subjects who worked with C-130 aircraft. For JP-8, distinct metabolite profiles were observed relative to controls for both F-15 and C-130 workers. Supervised Orthogonal Projection onto Latent Structures - Discriminant Analysis (OPLS-DA) confirmed that flight line subjects working with F-15s, regardless of jet fuel (JP-4 or JP-8), could be classified separately from control subjects with greater discriminating power and statistical confidence than C-130 flight line workers. Furthermore, it was also found that incorporating select metadata into the NMR spectral data analysis enhanced the discriminatory power and accuracy of the metabolomics analysis. Additional work is still required to identify key metabolites (biomarkers) that are predictive of exposure to specific jet fuels or their combustion products.

3.1 MATERIALS AND METHODS

3.2 *Subjects/Sampling*

The study was designed to recruit the following total number of flight line personnel subjects: 30 to 50 subjects from F-15 U.S. air base exposed to JP-8; 30 to 50 subjects from C-130 U.S. air base exposed to JP-8; 30 to 50 subjects from one or more F-15 JASDF air bases exposed to JP-4; 30 to 50 subjects from the C-130 JASDF air base exposed to JP-4. Control subjects needed to be from an airbase but away from the flight line activities. Since office personnel at JASDF air bases are exposed to jet fuel due to smaller sized bases and vapor that permeates the entire base, control subjects also needed to be sampled at Tachikawa Air Base where there are no flight operations. Office or hospital personnel were identified sampled in an identical manner as flight

line personnel: matched to the number of flight line personnel number (30 to 50) for JP-8 at U.S. air bases and matched to number of flight line personnel number (30 to 50) for JP-4 from JASDF air bases. Flight line subjects were volunteers who were active duty (USAF and JASDF) crew chiefs or other flight line personnel. Subjects could be male or female and the age range selected was between 18-50 years old. Subjects were questioned about the following information categories: Name (was in header but removed after sampling was completed); Career field; Rank; Years of service; Age; Gender; Work experience; Hobbies (other fuel or solvent exposures; worked on car or truck engines, either on or off duty); and Last time they fueled a government or personal vehicle and Type of fuel.

At end of shift questions were asked about exposures during shift as well as a brief summary of work activities associated with jet fuel and jet exhaust including time on flight line (location of job, physical activities). Additional questions were about exposure to any spills of any kind; any direct dermal exposure to jet fuel or any solvent or other exposures such as cleaning fluids; and if they were a smoker (if yes, number of cigarettes/cigars smoked). Samples were also obtained before and after second shift (~ 8 hr. shifts). Sampling days were Monday through Friday due to when subjects were available.

Prior to shift each subject provided a urine sample (entire void). See Appendix A for the Standard Operating Procedure for urine collection. The procedure was repeated post shift and the following morning. Urine weight was recorded and then 10 mL of urine each was placed into four vials plus 20 mL into another vial, frozen and stored frozen until analysis. Two 10 mL samples were for chemical analysis by Japanese Aero-Medical Laboratory (AML) to analyze for jet fuel components. The two 10 mL and the 20 mL frozen samples for biomarker analysis were shipped to 711 HPW/RHDJ at WPAFB.

Prior to the shift sampling, each subject had a personal air monitor (PAS) placed upon them where the PAS 500 was positioned in a pocket of a vest worn by the subject. The monitor went into the chest pocket with just the tip of the charcoal tube extending from the pocket. Prior to their shift, each subject also had a Sioutas Personal Cascade Impactor (SPCI) particle monitor clipped on to the vest with the pump clipped on to the belt of the vest. Both monitors were removed after the shift for laboratory blood and urine analysis for volatile organic compounds (VOCs) by Japanese collaborators.

3.3 *Urine Samples*

Subjects provided urine samples prior to work shift (6-8 AM), post-shift (4-6 PM), and 12-16 h post-shift (6-8 AM). Urine volumes were recorded and 10 mL of urine were placed into each of four vials plus 20 mL into another vial, frozen, and stored frozen until analysis. Two 10 mL samples were used for chemical analysis by AML to analyze for jet fuel components. The

remaining two 10 mL and the 20 mL frozen samples were shipped to 711 HPW/RHDJ. The urine samples were transported to WPAFB on dry-ice in an insulated pack. Upon arrival at WPAFB, all urine samples were stored at -80 °C until assayed by NMR or LC/MS spectroscopy. Prior to NMR analysis, frozen urine samples were thawed at 4 ^{o}C overnight. A 600 μl aliquot of urine was then mixed with 300 μl of phosphate buffer (0.2 M mono- and disodium phosphate; pH 7.4) and centrifuged at 13,000 rpm for 10 min to remove any precipitates. A 550 μl aliquot of the supernatant was transferred to a 5 mm NMR tube and mixed with 150 μl of 2,2',3,3'–tetradeutero-trimethylsilylpropionic acid (TSP) in deuterium oxide (D_2O), adjusted to yield a final concentration of 2 mM. The TSP served as a chemical shift reference (δ = 0.00 ppm) and quantification standard, and D_2O provided a field-frequency lock for NMR data acquisition. Urine samples were screened for all detectable changes/possible biomarkers by the methods developed by RHDJ for identifying biomarkers in urine using LC-MS (Shiyanov *et al*., 2009) and 600 mHz NMR (DelRaso, *et al*., 2009)

3.4 *NMR Spectroscopy Data Acquisition*

Proton NMR spectra were acquired at 25 °C on a Varian INOVA NMR instrument operating at 600 MHz. Water suppression was achieved using the first increment of a Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence, which incorporates saturating irradiation (on resonance for water) during the relaxation delay (7.0 s total; 2 s with water pre-saturation) and the mixing time (50 ms total; 42 ms with water irradiation). Data were signal averaged over 128 transients using a 4.0 s acquisition time and interpulse delay of 11.05 s.

NMR spectral data were processed using Varian software (VNMR 6.1c) and employing exponential multiplication (0.3 Hz line-broadening), Fourier transformation, and baseline flattening (fifth-order polynomial and spline fitting routines). Spectra were then baseline corrected (flattened) in MATLAB (The Mathworks, Inc. Natick, MA; v. R2010b) using the Whittaker Smoother algorithm (with lambda value of 200) on selected spectral noise regions (Eilers 2003; Whittaker 1923). Spectral resonances in urine from TSP (0.0 ppm), residual water (4.72-5.00 ppm) and urea (5.54-6.01 ppm) were excluded from the analyses. Spectra were then preprocessed using a probabilistic quotient normalization (PQN) method. This method is based on the calculation of a most probable dilution factor by looking at the distribution of the quotients of the amplitudes of a test spectrum by those of a reference spectrum (Dieterle et al., 2006). To reduce the dimensionality and mitigate peak misalignment, a dynamic programming-based adaptive binning technique was employed (Anderson et al. 2011) using a minimum and maximum distance between peaks in a single bin of 0.001 and 0.04 ppm, respectively. Bin boundaries were then manually adjusted to further mitigate peak misalignment, and to keep known J-coupled multiplets within that same bin (e.g., doublets, triplets, etc.). Integrated bin areas were transferred to an Excel file and normalized to the TSP signal intensity. Data were then autoscaled using various datasets as reference.

3.5 *NMR Data Processing*

Multivariate data analyses were conducted on binned, scaled spectral data using MATLAB software constructed in-house (Anderson et al. 2011). Binned NMR data were scaled to a chosen reference dataset by subtracting each bin value from the mean value for the corresponding bin in the reference data, then dividing this value by the standard deviation of the reference data (auto-scaling).

Principal Component Analysis (PCA), applying no prior knowledge of human experimental data acquisition control for the NMR binned data, is an unsupervised statistical procedure using orthogonal transformation to convert the possibly correlated NMR bin set variables into a set of values of linearly uncorrelated variables called principal components based upon data variance; PCA provided a first analysis approach for data visualization. As previously described (Mahle et al. 2010), PCA model constructs were based on specific experimental groups to explore any systematic differences between groups that may exist. Once the model is constructed, other groups can then be superimposed into the visualization, by applying the model-specific bin coefficients (PCA loadings), to show how they compare. Thus PCA models were constructed to maximize visualization of specific data features based upon the nature of the effects being assessed, and PCA scores plots were used to help identify the time points of maximum effects for treatments.

OPLS-DA was used as a supervised technique to classify data and identify salient features that allow class separation (Wold et al. 2001). In order to apply OPLS-DA, spectral data were collected into a matrix of variables or bins (X) and a vector of categorical labels (Y), representing the effects. These data were then analyzed and modeled as follows: (1) determine a specific time point of interest; (2) encode each treatment and corresponding control group as a two-group problem and analyze with OPLS; (3) using the model created for this specific two-group problem, project the remaining samples from other groups into the OPLS model. Therefore, OPLS enabled classification into specific groups. The OPLS model was evaluated on its predictive ability, using the Q^2 (coefficient of prediction) metric. Q^2 was calculated as follows:

$$Q^2 = 1 - \frac{PRESS}{SSY} = 1 - \frac{\sum_{i=1}^{n} e_i^{\,2}}{\sum_{i=1}^{n} (y_i - y_i)^2} = 1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \overline{y_i})^2}$$

where PRESS is the Predicted Residual Sum of Squares calculated as the residual e_i between the predicted and actual Y (class labels) during leave-one-out cross-validation, SSY is the Sum of Squares for *y*, $\bar{y}$ is the *y* mean across all samples, and $\hat{y}_i$ is the *y* value for i^{th} sample. As Q^2 approaches 1, the more predictive capability the model exhibits. A Q^2 value less than zero indicate that the model has no predictive power. A permutation test was performed to evaluate the significance of the Q^2 metric. The test involved repeatedly permuting the data labels and re-running the discrimination analysis, resulting in a distribution of the Q^2 scores (Westerhuis et al. 2008). The Q^2 from the correctly labeled data is then compared to the distribution to determine the significance of the model at a specified alpha (set herein at $\alpha = 0.01$).

Variable selection (salient bins) from OPLS-DA was also statistically evaluated. The bin loadings, commonly referred to as coefficients, were compared to calculated null distributions in order to select for significance. The null distribution for each bin was determined by refitting the OPLS model to datasets in which each bin was independently and randomly permuted to remove any correlation between it and the control/treatment groups. The true OPLS model loading was then compared to the resulting null distribution of loadings, and values in the tail (greater than 99.5% or less than 0.5% of the null distribution; corresponding to $\alpha = 0.01$) were assumed to contribute significantly to the model. The permutation was initially repeated 500 times for each bin and those near-significant loadings (greater than 92.5% or less than 7.5% of the null distribution; corresponding to $\alpha = 0.15$) were selected for 500 additional permutations (total 1000). Comparisons between individuals were used to help identify metabolite profiles or novel chemical exposure markers. The salient spectral resonances were assigned to metabolites using Chenomx 5.1 software, on-line NMR databases (i.e., mmcd.nmrfam.wisc.edu; U. Wisc, etc.).

4.1 RESULTS

4.2 *Metabolomics Urine Sample Collection*

Urine samples were collected from non-flight line and flight line personnel from both Japanese (3) and U.S. (2) air bases located in Japan. Both Japanese and U.S. bases involved C-130 and F-15 aircraft flight operations. However, Japanese air bases utilized jet propulsion type 4 fuel (JP-4) for their aircraft while U.S. air bases utilized JP-8. Human urine sample were provided prior to the start of a work day (pre-shift), at the completion of a work shift (post shift-1) and 12-16 h following the completion of a work shift (post shift 2). Proton NMR spectra were acquired from 343 urine samples using a 600 mHz Varian NMR spectrometer. A summary of the urine sample collections from the various bases is shown in Table 1.

TABLE 1. Sample information for jet fuel study showing the number of samples received for Japanese and US personnel at various air bases, with identification of the specific operational aircraft.

Air Base	Nationality	Fuel	Aircraft	Pre-Shift (N-value)	Post-Shift (N-value)
Komaki (Ki)	Japanese	JP4	C130	16	16
Komatsu (Ku)	Japanese	JP4	F-15	19	19
Naha C (Nh)	Japanese	JP4	F-15	14	29
Naha C (Nh)	Japanese	Control	–	20	21
Kadena (Kd)	US	Control	–	24	23
Kadena (Kd)	US	JP8	F-15	33	33
Yokoto (Yo)	US	Control	–	22	22
Yokoto (Yo)	US	JP8	C-130	16	16
		Total #samples =		**164**	**179**

Initially, all spectra were reprocessed such that all parameters were identical across the complete dataset of 343 samples. However, it was later discovered that the spectral data needed to be re-binned to increase the resolution in the binned spectral dataset. Some of the bin widths in our original processing of the data were too broad and the total number of bins across the spectral bandwidth was approximately 125 bins. Thus, we decided to re-bin these data to increase resolution in the analysis. After completing this processing, each spectrum represented approximately 250 bins.

The resulting datasets were then scaled using different subject groups as reference; each scaling procedure created a new dataset with a specific reference (i.e., autoscale using all controls [non-flight line] as reference). Scaling the data is an important processing procedure and can yield different results. Thus, as an initial step in data processing multiple scaled datasets were prepared and principal components analysis (PCA) was conducted to examine which scaling procedures yield the best subject group separation.

4.3 *PCA of NMR Data from Japanese Subjects at Pre- & Post-Shift Times.*

All Japanese control subjects were derived from one of the Japanese airbases (Naha; Nh). Principal component analysis (PCA) of Japanese control subject urine samples at pre-shift and post-shift times indicated metabolite profile differences (Fig. 1). This finding was not unexpected as the first urinary void in the morning would likely show a different profile relative to others throughout the day during periods of activity and eating. However, this finding suggests that care

should be taken when making comparisons between flight line exposed subjects and control subjects.

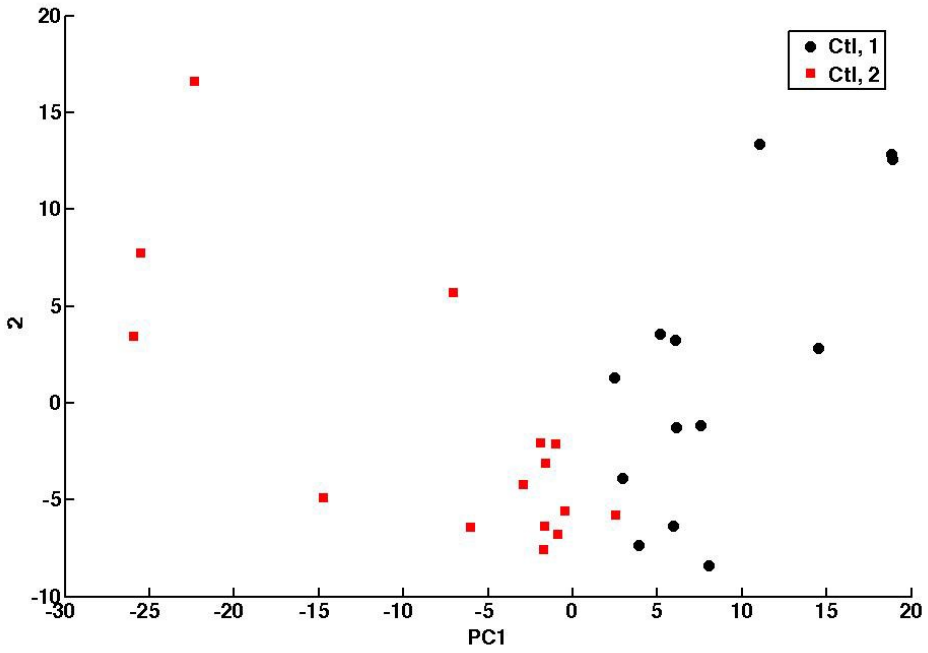

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Figure 1. PCA scores plot (PC1 *vs.* PC2) for Japanese controls at Nh airbase showing urine collected at pre-shift (Ctl 1; black) and post-shift (Ctl 2; red) times. Data were autoscaled using all JP-4-exposed subjects as reference (pre- & post-shift times).

A similar PCA analysis was conducted using the Japanese flight line personnel potentially exposed to JP-4. PCA analysis of Japanese subjects from all air bases (Nh, Ku and Ki) indicated considerable overlap in urinary metabolite profiles (Fig. 2). This result suggests that urinary metabolite profiles from pre-shift subjects may reflect prior daily flight line exposures.

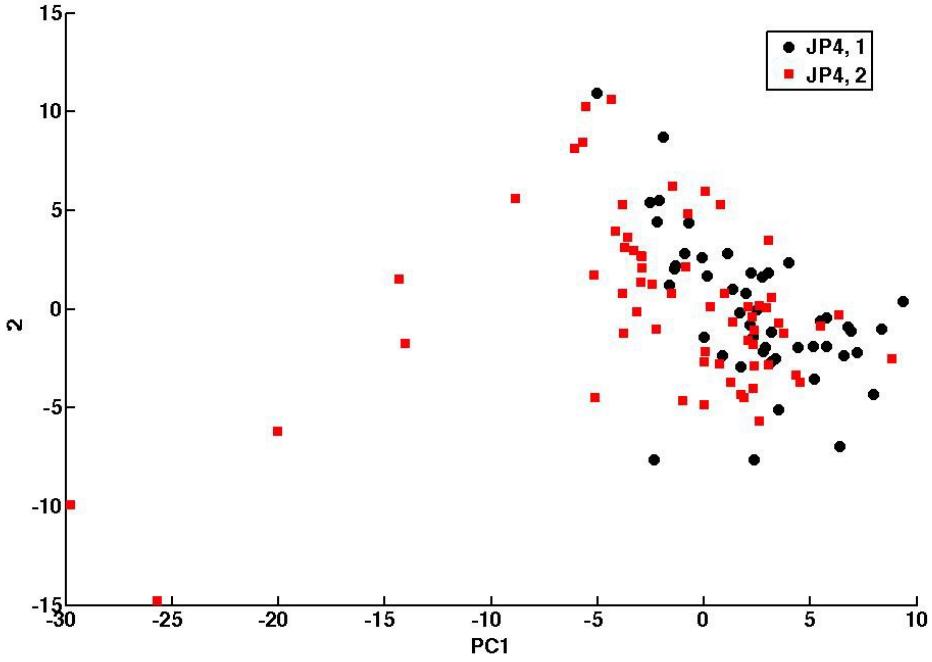

Figure 2. PCA scores plot (PC1 *vs.* PC2) for all Japanese flight line subjects potentially exposed to JP-4 showing NMR urine analysis at pre-shift (black) and post-shift (red) times. Subjects were flight-line workers at Nh, Ki and Ku airbases. Data were autoscaled using all flight line subjects (pre & post-shift) as reference.

PCA analysis was also performed on Japanese subjects at each air base separately. Two of the three Japanese bases (Nh and Ku) involved F-15 flight operations while the remaining air base (Ki) involved C-130 flight operations. PCA analysis of pre- and post-shift urine samples from flight line subjects at Ki air base (C-130 aircraft) indicated significant overlap (Fig. 3). Although overlap with control subjects was observed in the urinary profiles obtained from flight line personnel located at the F-15 flight operation bases (Nh and Ku), there appeared to be a greater urinary metabolite profile separation from control with flight line personnel located at Ku air base (Fig. 4).

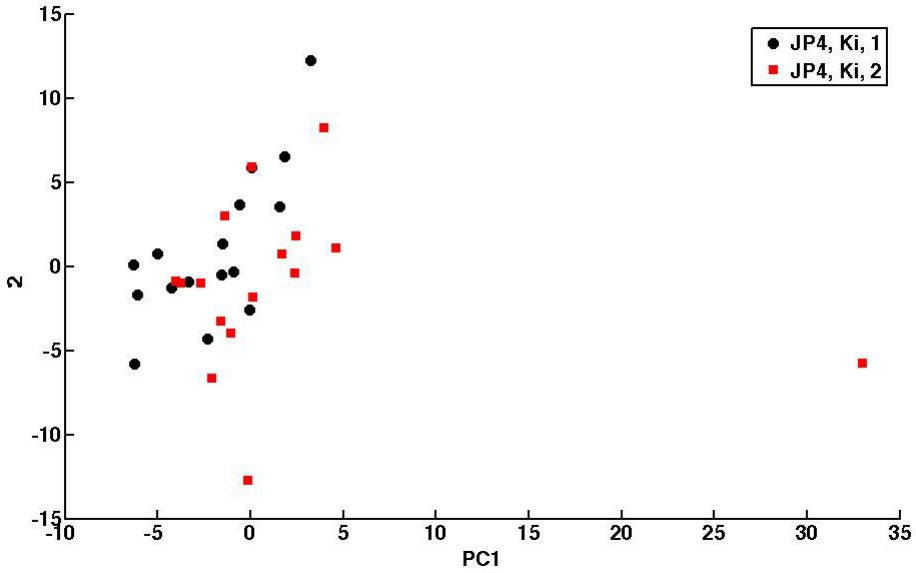

Figure 3. PCA scores plot (PC1 *vs.* PC2) for flight line personnel potentially exposed to JP-4 while working on the flight line at Ki (C-130) airbase. Urine samples were collected at pre-shift (black) and post-shift (red) times. Data were autoscaled using all Japanese flight line subjects (pre- & post-shift times).

Overall, there appeared to be greater separation in the urine metabolite profiles of the control group between pre-shift and post-shift time points (compare with Fig. 1). This result further suggests prior jet fuel exposures in flight line personnel resulting in a more similar urinary metabolite profile between pre- and post-shift samples.

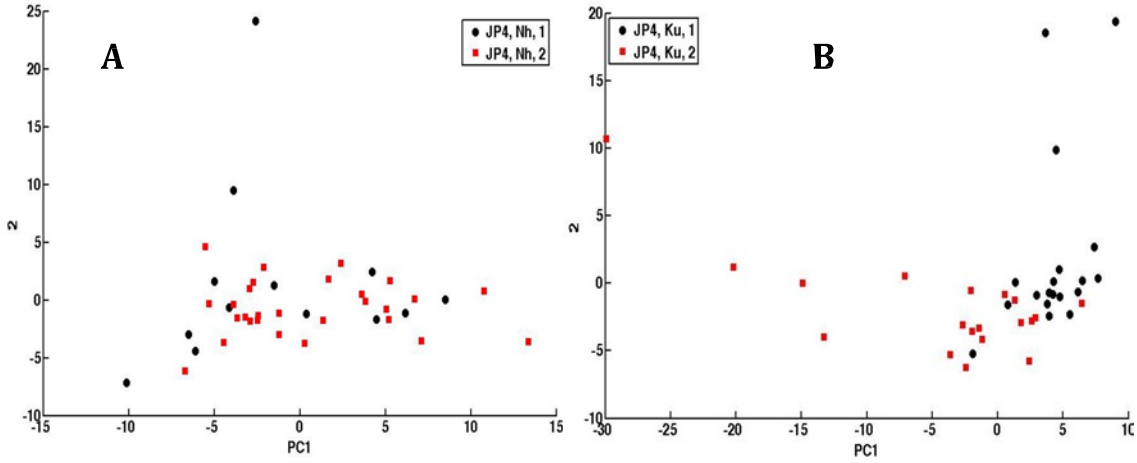

Figure 4. PCA scores plots (PC1 *vs.* PC2) for JP-4-exposed subjects refueling F-15 aircraft at Nh (A) and Ku (B) airbases. Urine samples were collected at pre-shift (black) and post-shift (red) times. Data were autoscaled using all JP-4-exposed subjects as reference (pre & post-shift times).

4.4 *Paired-By NMR Data Analysis*

NMR spectral data can be 'paired' to emphasize difference in the pre- *vs.* post-shift urine samples. Pre-processing NMR data in this manner provides a difference spectrum (post – pre) that can then be used as input to PCA. This type of NMR data analysis provides discovery of changes in metabolite profiles over the work-shift time course. Paired analyses of NMR urinary spectral data obtained from Japanese subjects working at Japanese air bases servicing JP-4 fueled aircraft (C-130 and F-15) were found to yield the best data separation results for this dataset. The PCA results for control and Japanese flight line personnel potentially exposed to JP-4 were conducted separately for each air base and are shown in Figure 5. Paired analysis of urine samples from flight line personnel appeared to indicate that personnel who worked at air bases involved with F-15 flight operations showed the greatest urinary metabolite profile separation when compared to control subjects (Fig. 5B and C).

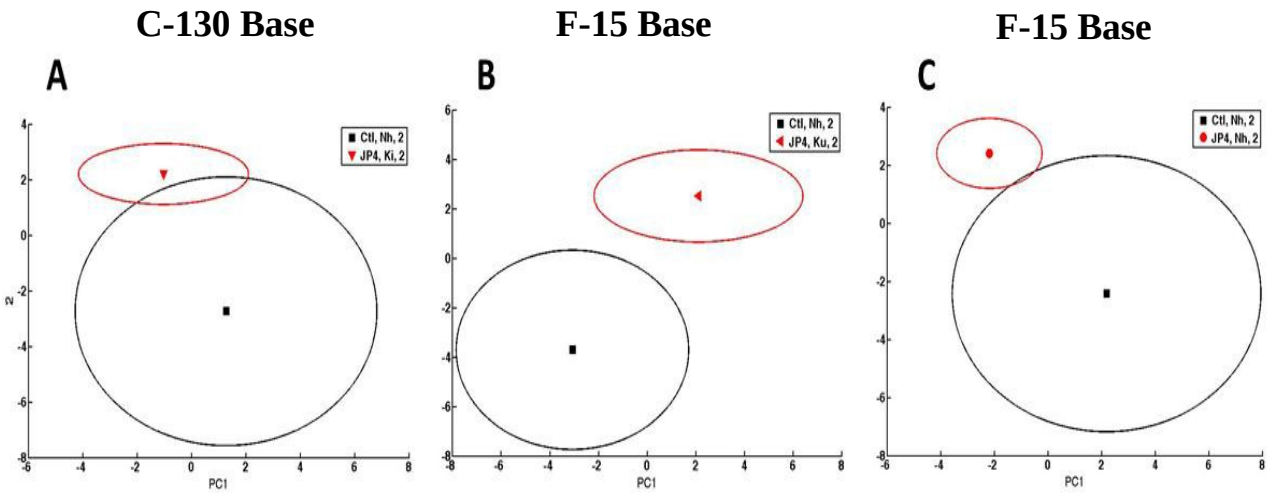

Figure 5. PCA scores plots (PC1 *vs.* PC2) showing control (black) and flight line JP-4 potentially exposed (red) subjects at Ki (A), Ku (B), and Nh (C) airbases. Data were autoscaled using all flight line subjects as reference (pre- + post-shift times). Input data to PCA were paired-by the pre-shift time for each subject to emphasize the change in urinary metabolites during the course of a work-shift. Plots show the centroid Mean ± 2SEM.

These findings suggested that not only were there observable differences in urine metabolite profiles between control and flight line personnel, but also there appeared to be a significant difference in the urine profiles of flight line personnel between those involved with C-130 flight operations and those involved with F-15 flight operations. Indeed, when PCA was performed on NMR data derived from flight line personnel grouped by type of operational aircraft, a significant difference was observed (Fig. 6). Clearly, urine profiles derived from flight line

personnel working F-15 air bases were more differential than those working C-130 air bases when compared to control. These NMR metabolite profile data seem to imply that airframe JP-4 combustion product exposure may be more predictive of flight line exposures than exposure to the JP-4 fuel itself, since the volume of un-combusted fuel moved is higher at C-130 airbases. This idea also assumes that fuel handling practices and safety are equivalent between F-15 and C-130 bases.

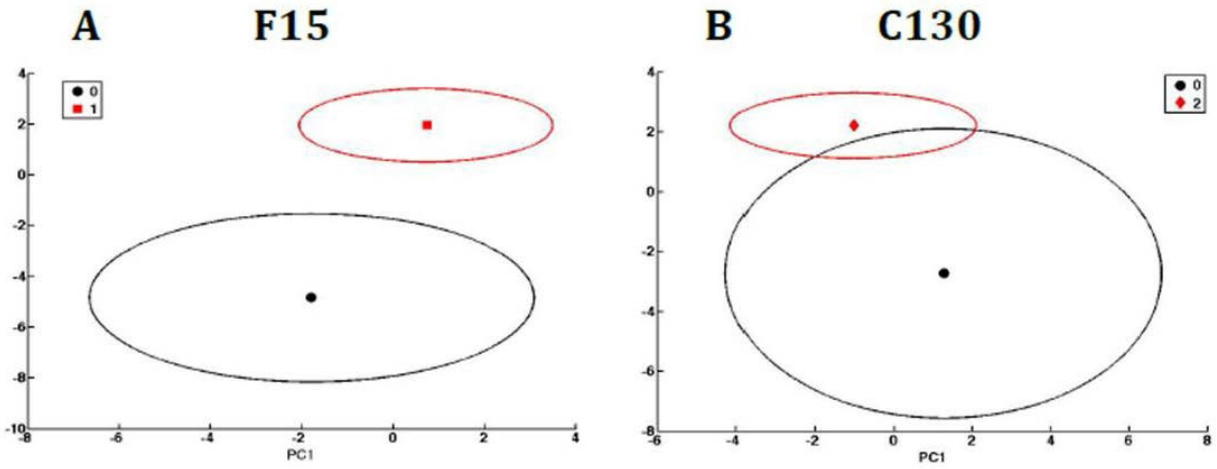

Figure 6. PCA scores plots (PC1 *vs.* PC2) showing control (black) and flight line JP-4 potentially exposed (red) subjects working with F-15 (A) or C-130 (B) aircrafts (centroid mean ± 2SEM). All controls were stationed at Nh airbase. F-15 flight line subjects were at Nh and Ku bases, while C-130 flight line subjects were at Ki airbase. Data were autoscaled using all flight line subjects as reference (pre- & post-shift times). Input data to PCA were paired-by the pre-shift time for each subject to emphasize changes in urinary metabolites during the course of a work-shift.

A PCA was also conducted in which controls and all flight line exposed personnel from all bases (Na, Ki and Ku) were included in the same PCA model analysis. The resulting PCA scores plot clearly shows distinct urinary metabolite profiles between control and flight line personnel regardless of the type of aircraft serviced (Fig. 7). These data suggest that there may be a specific set of urinary metabolite features that are associated with exposure to JP-4 or combustion products of JP-4.

4.4. *Discriminant Analysis of Control vs. JP-4-Exposed Subjects.*

A supervised 'orthogonal projections onto latent structures' discriminant analysis (OPLS-DA) using the paired datasets was performed to determine whether control subjects and flight line

subjects could be classified separately. This analysis also enabled us to determine whether the group classification is statistically significant. If significant, the loadings (coefficients) from the OPLS-DA can be used to identify the salient features (spectral metabolite signals) that lead to group separation.

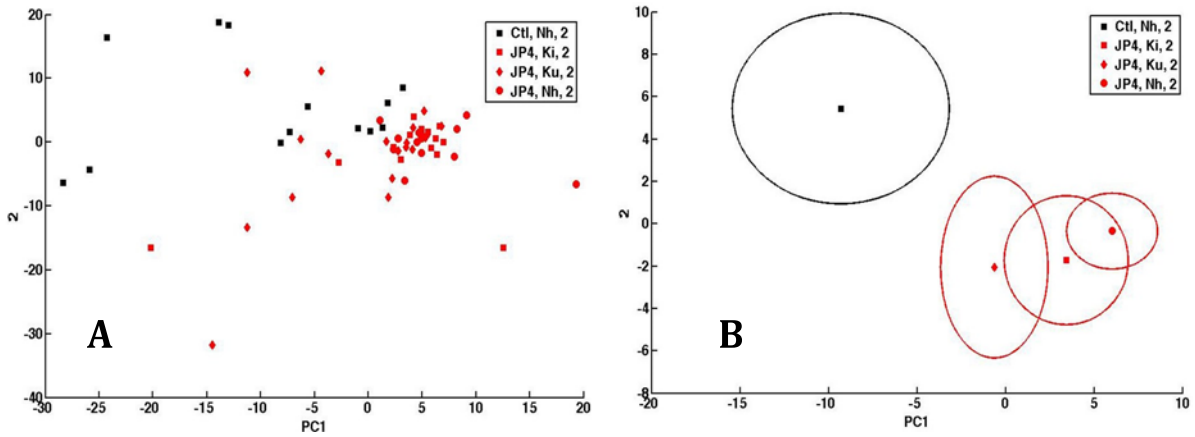

Figure 7. PCA scores plots (PC1 *vs.* PC2) showing control (black) and flight line JP-4 potentially exposed (red) subjects at Ki (square), Nh (circle), and Ku (diamond) airbases. (A) Displays data for individual subjects in each group, while (B) shows the centroid mean ± 2SEM for each group. Input data to PCA were paired-by the pre-shift time for each subject. Data were autoscaled using all flight line subjects as reference (pre- & post-shift times).

To examine whether subjects could be classified separately based on flight line-exposure, and whether results were truly more significant for personnel working F-15 *vs.* C-130 operational air bases, OPLS-DA was conducted on NMR data. OPLS-DA of NMR data derived from urine samples obtained from Japanese flight line personnel working at Ki air base (C-130) indicated statistically significant differences ($Q^2 = 0.270$, $p=0.01$) in urine metabolite profile when compared to control, but the Q^2 (coefficient of prediction) was much lower than that derived for personnel working F-15 operational bases (Fig. 8A). OPLS-DA conducted on NMR data obtained from personnel working at F-15 operational bases (Ku and Nh) yielded more significant Q^2 values of 0.418 and 0.589, respectively ($p < 0.01$; Fig. 8B and C). Finally, NMR data from personnel at both Ku and Nh (F-15 bases) were combined and OPLS-DA was performed. Results indicated statistical significance yielding a Q^2 value of 0.514 and predictive accuracy (leave-one-out cross validation) of 100% (Fig. 8D). All statistical analyses were conducted by computing a Q^2 distribution for 500 permutations of data labels.

The OPLS-DA also provided a listing of the significant bins important for group classification; a bin is a small region of the NMR spectrum containing metabolite resonances. Table 2 identifies

each significant bin by its center positions (δ in ppm) and the listing is rank-ordered from most-to-least important (|P| value). The JP-4/C-130 and JP-4/F-15 datasets yielded 13 and 15 salient features, respectively, that were important for their group classification. Four of these features were shared in both datasets (highlighted in yellow). Work is currently underway to identify the specific urinary metabolites that are represented in these bins, and to examine how these metabolites may be related to flight line exposures at Japanese operational air bases utilizing JP-4 fueled aircraft.

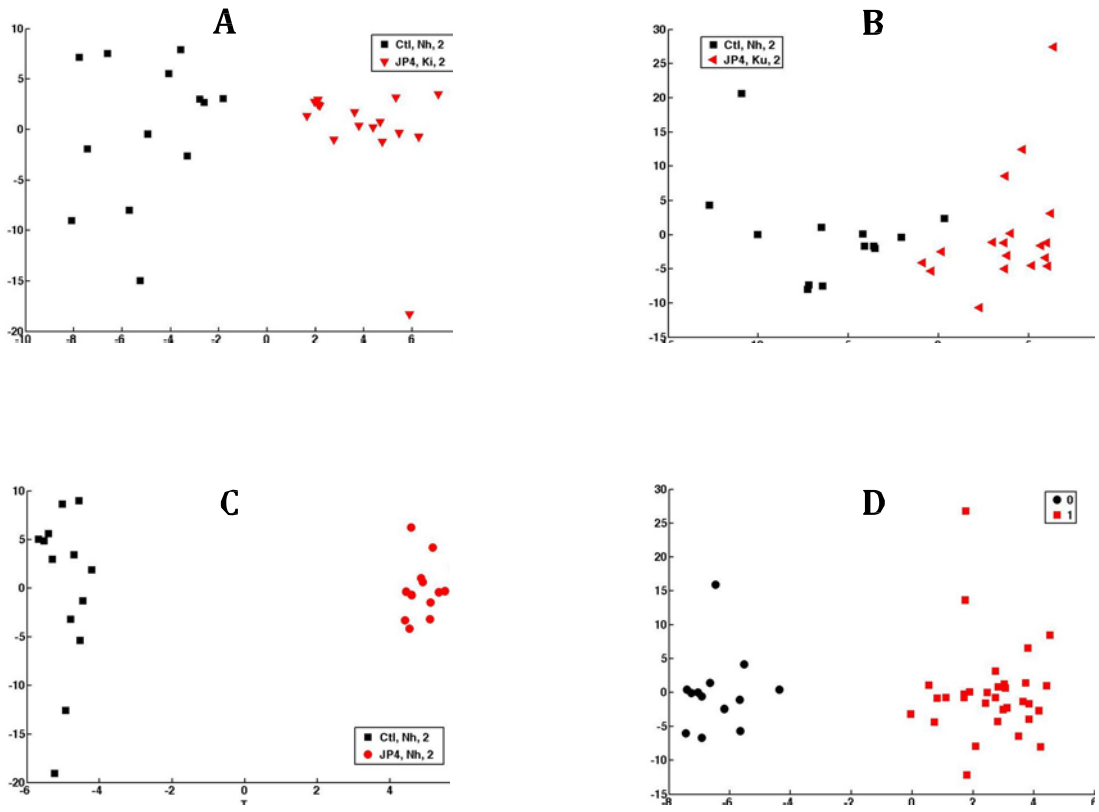

Figure 8. T-score plots derived from OPLS-DA using 'paired data' for control (black) *vs.* flight line JP-4-potentially exposed (red) subjects. Data were autoscaled using all flight line subjects as reference (pre + post-shift times), and paired-by the pre-shift time for each subject. Analyses were conducted for each airbase separately: (A) Ki, (B) Ku, and (C) Nh. A separate analysis examined control *vs.* flight line subjects working with F-15 aircraft only (D). All results are statistically significant ($p \leq 0.01$). See text for details.

Table 2. Listing of significant bins from OPLS-DA for Control *vs.* JP4-C130 and Control *vs.* JP4-F15 analyses. Bin locations (chemical shift, δ) are identified by the center position for each bin. Data are listed in rank-order for each analysis (highest to lowest |P| value). Common features found in both analyses are highlighted in yellow.

Control *vs.* JP4-C130 (Q^2 = 0.270; p<0.01)		Control *vs.* JP4-F15 (Q^2 = 0.514; p<0.01)	
δ (ppm)	|P|	δ (ppm)	|P|
1.488	0.234982	4.654	0.270036
2.002	0.192924	4.563	0.217502
5.477	0.170652	4.594	0.196708
2.017	0.147214	2.235	0.156112
4.404	0.141868	1.258	0.139695
0.645	0.111085	2.017	0.132464
1.113	0.108162	2.002	0.131589
1.795	0.105954	2.102	0.117800
5.188	0.100399	4.375	0.117756
5.128	0.097265	5.128	0.109806
3.667	0.070811	1.222	0.109545
5.161	0.062185	1.247	0.103986
1.239	0.058489	1.239	0.099685
		2.565	0.075586
		2.378	0.054582

4.5 *PCA of U.S. Subjects at Pre- & Post-Shift Times.*

Unlike the Japanese urinary metabolomics dataset, control urine samples from U.S. personnel at two air bases in Japan (Kadena [Kd] and Yakota [Yo]) were obtainable from both air bases. Two U.S. subjects yielded spectra in which the baselines were distorted and could not be properly corrected. Therefore, these data were discarded (Kd3-post and Kd17-pre). Data scaling was similar to the procedures used for the Japanese subject dataset, multiple autoscaled datasets were constructed and PCA was performed to search for the best scaling procedure to separate flight line personnel potentially exposed to JP-8 from controls. In contrast to Japanese personnel, optimal statistical analysis for U.S. personnel was achieved when datasets were autoscaled using the control group (all controls, pre- + post-shift times) as reference. This was believed to be due to personnel scheduling differences between U.S. and Japanese flight line personnel (i.e. Japanese personnel may have longer off-times prior to scheduled shifts). If U.S. personnel were working consecutive daily shifts, then paired data analysis would not be possible because pre-shift samples would closely resemble post-shift analysis depending on the length of time between scheduled shifts.

Similar to Japanese control subjects, PCA analysis of urinary NMR data obtained from control subjects located at Kd and Yo collected pre-shift and post-shift indicated significant differences (Fig. 9). Since paired data (post- minus pre-shift times) did not yield good separation in PCA space, PCA was conducted for all U.S. subjects including pre- and post-shift times. Two of the

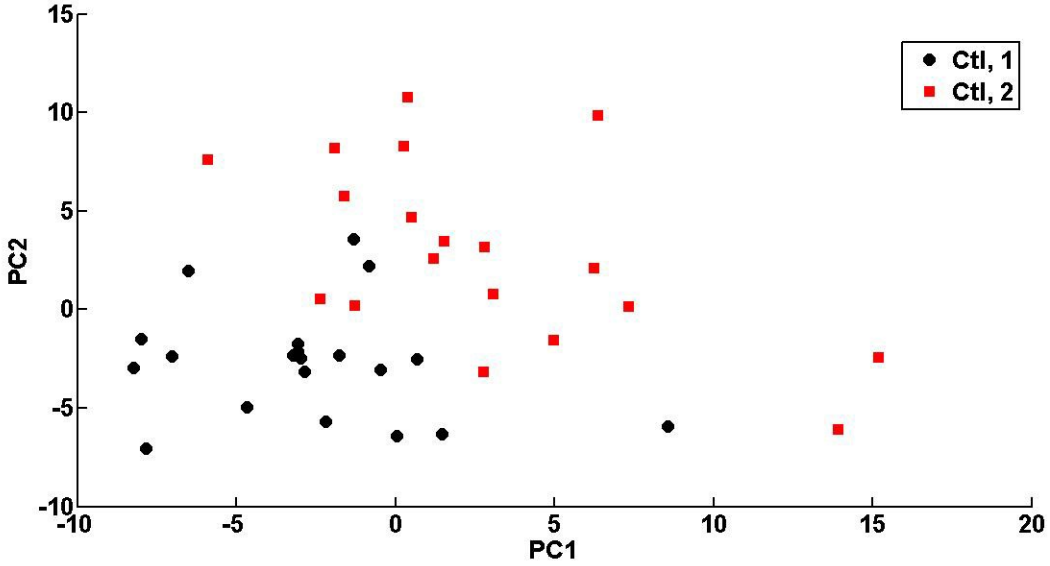

Figure 9. PCA scores plot (PC1 *vs.* PC2) for U.S. controls at Kd and Yo air bases from urine samples collected pre-shift (black) and post-shift (red). Data were autoscaled to the all controls (pre- & post-shift times).

flightline subjects (Kd30 and Yo7) were identified as outliers. Interestingly, both of these subjects reported spill-exposures to JP-8 and hydraulic fluid on their study questionnaires, respectively. Urinary NMR analysis for each of these subject outliers was clearly displaced, relative to other data, in PCA space at both pre- and post-shift time points and may be the result of exposure to these spilled hydrocarbon-based fluids (Fig. 10A). These outliers impart a large degree of variability within the flight line subject dataset causing the control and flight line subject PCA data to overlap at the 95% confidence interval (CI; Fig. 10B). Because these outliers dominate the analysis, they may mask differences in metabolite profiles between control and flight line groups that exist for other subjects. Therefore, subjects Kd30 and Yo7 were omitted in further analyses. Re-analysis of the data found that many of the flight line subjects appeared to separate from control subjects (Fig. 10C). Indeed, the PCA scores plots displaying ellipsoid means (±2SE; 95% CI) for each cohort group showed clear separation (Fig. 10D). However, there were many subjects within the fight line group that overlapped with subjects in the control group. This finding indicated that there was significant variability among flight line workers and may relate to the amount of time spent on the flight line. These data were further examined with regard to the influence of type of aircraft (C-130 or F-15) operating at a particular air base involving personnel working the flight line. U.S. subject urine samples from personnel working C-130 (Yo) and F-15 (Kd) air bases were identified and analyzed by PCA (Fig. 10E and F). Interestingly, the variability in urine metabolite profiles of U.S. personnel working the F-15 flight line (Kd) was much lower than that of the U.S. personnel working the C-130 flight line (Yo) indicating potential differences in flight line exposure levels between each type of aircraft fueled with JP-8 (Fig 10E).

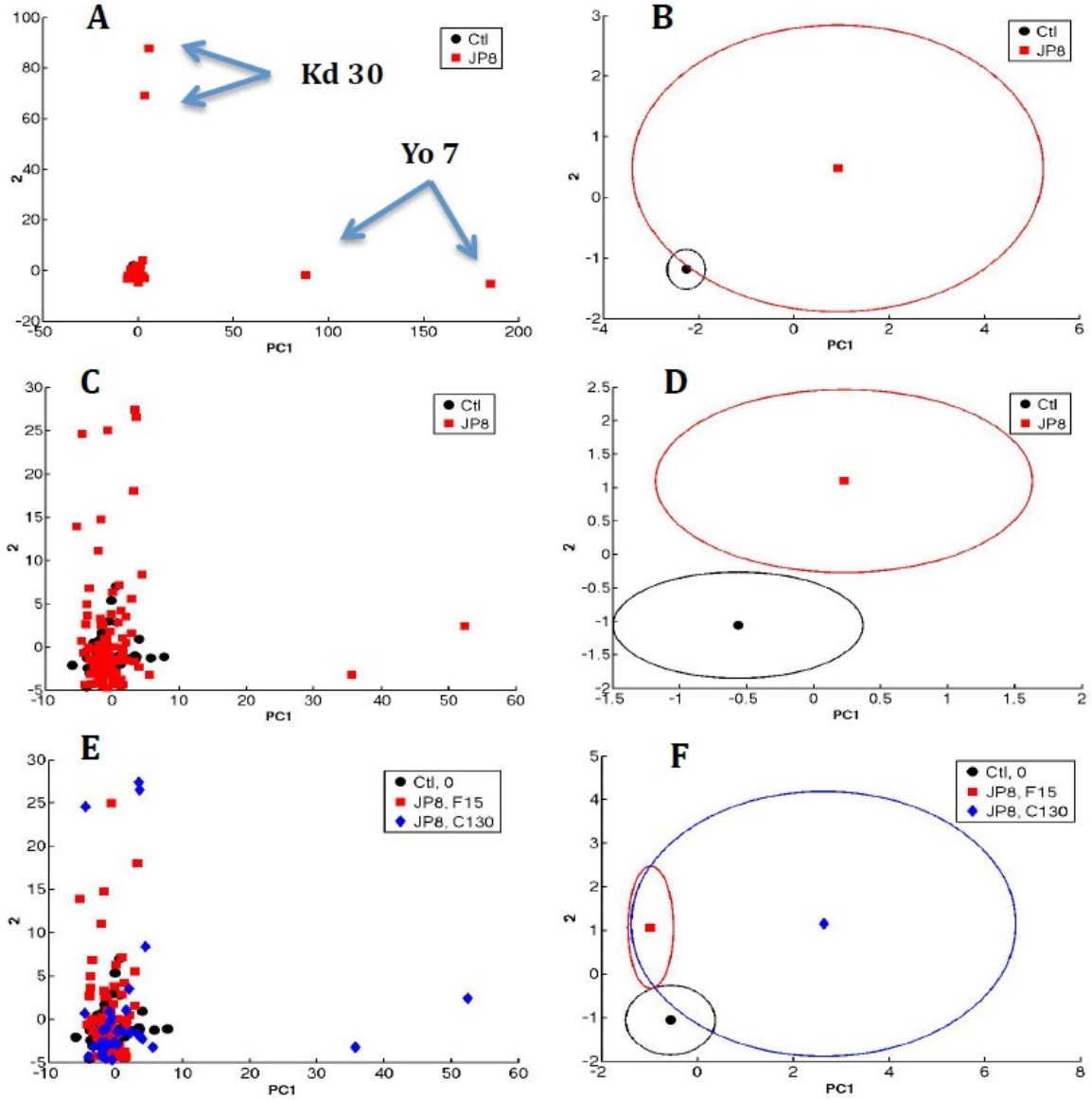

Figure 10. PCA scores plots (PC1 vs. PC2) for control (black) and flight line JP-8 potentially exposed (red and blue) U.S. personnel at Kd and Yo air bases showing all subjects (A) and the centroid Mean ± 2SEM (B). Data were autoscaled using the control subjects as reference (both Kd and Yo air bases and pre- & post-shift times). Note that Yo7 and Kd30 are outliers (A) and cause a large variability in the flight line group (B). When these two subjects are omitted from the analysis, then other flight line subjects appear to separate from controls (C) and the groups cluster separately in the Mean ± 2SEM plot (95% CI; D). An identical analysis where flight line subjects working with F-15 (red) and C-130 (blue) aircrafts were identified yielded scores plots displaying individual subjects (E) and Mean ± 2SEM (95% CI; F).

4.6 *Discriminant Analysis of Control vs. JP-8-Exposed Subjects.*

Following PCA analysis, OPLS-DA was conducted to determine if flight line personnel could be distinguished from control with statistical significance based on their urinary metabolite profiles. If OPLS-DA is conducted with the inclusion of the two subject outliers (Kd30 and Yo7), significant classification is achieved, but the predictive coefficient is low (Q^2 = 0.169; data not shown). If the two subject outliers are removed from OPLS-DA, and each air base data from human urine is analyzed separately (i.e. control vs. F-15 flight line personnel and control vs. C-130 flight line personnel), statistically significant classification ($p < 0.01$) is achieved between control and flight line personnel at both air bases (Fig. 11). However, the predictive coefficient for the flight line personnel working the F-15 air base (Kd) was greater (Q^2 = 0.408) than that for personnel working the C-130 airbase (Yo; Q^2 = 0.149).

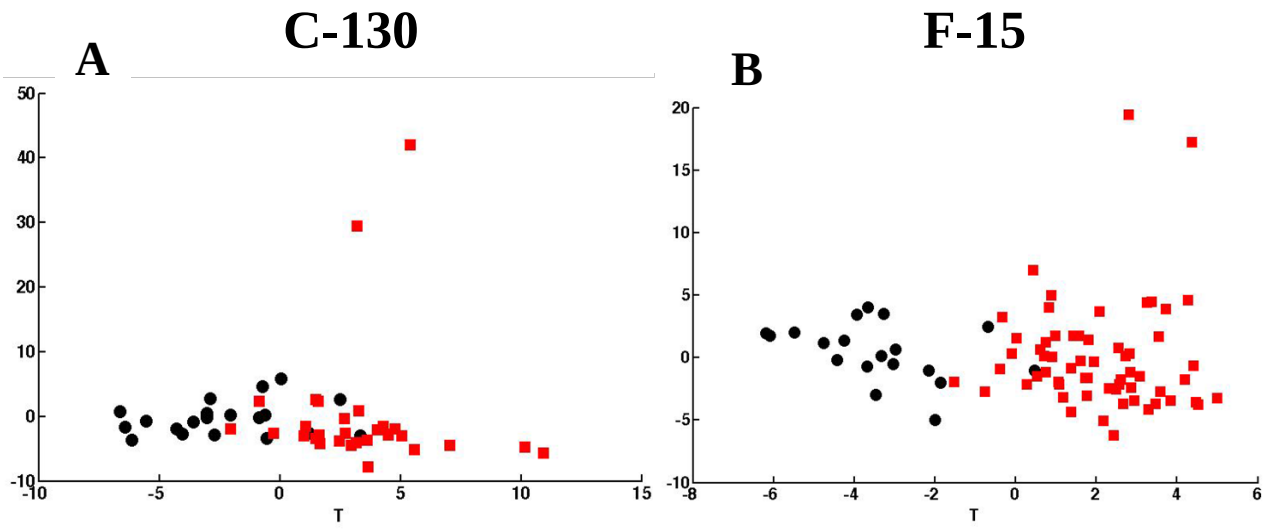

Figure 11. OPLS-DA T-score plots for control (black) vs. flight line JP-8 potentially exposed subjects (red) with Yo7 and Kd30 excluded. Analyses were separated by aircraft, and all data were autoscaled using the control subjects as reference (both Kd and Yo air bases and pre- & post-shift times). Flight line subjects working at C-130 (A) and F-15 (B) air bases can each be classified separately from controls with Q^2 values of 0.149 and 0.408, respectively ($p<0.01$).

4.7 *Calculation of an Apparent Exposure Index (AEI) for Enhanced NMR Analysis*

Metadata for U.S. and Japanese personnel were collected for this study. These metadata included, but not limited to, various information about each subjects' working environment, known chemical exposures, and measured exposure to VOCs during their work shift. The VOCs were measured by two methods (charcoal filter and ORBO Cascade Impactor). Metadata was used in conjunction with NMR data to determine if inclusion of metadata would enhance PCA group classification predictability based on urinary metabolite profile. Since this is a relatively new concept for metabolomics data analysis, only metadata for U.S. personnel were used as a proof-of-concept. We determined the most relevant metadata information that may influence NMR spectra and used these data to construct an Apparent Exposure Index (AEI) because there was no consistency with regards to the number of hours worked on the flight line by each subject. Four categories of metadata were chosen that were believed to have the most influence on human exposure impact and thereby NMR spectral data: 1) average time on the flight line, 2) exposure to spills, 3) inhalation exposure (primarily exhaust), and 4) total measured VOCs.

The total VOC value was calculated by readjusting the VOC data obtained by the two measurement methods. This readjustment was necessary since two devices were used to obtain VOC values, and some subjects had values from only one method (charcoal or ORBO), while others had values from both methods. VOC data were normalized by the following procedure: 1) The range of VOC measurements by both methods yielded similar results (~ 0 – 73 ppb) and thus, the normalization value was set at 73 ppb (each ppb value was then divided by 73), 2) If a subject had a VOC measurement by each method, the average value was normalized, and 3) Normalized VOC values were multiplied by 10 to yield final VOC index score. The remaining three metadata category index scores were derived arbitrarily as indicated in Table 3. The final AEI was equal to the sum of assigned index scores for each category.

Table 3. Metadata category system devised to calculate AEI

Category	*If* the Value =	*Then* the Assigned Score =
Average Time on Flight-line	0 h	0
	1 – 6 h	1.0
	6 – 12 h	1.5
Exposure to Spills	none	0
	Yes (other than JP8)	1.0
	Yes (JP8)	1.5
Inhalation Exposure	none	0
	Yes (other than JP8)	1.0
	Yes (JP8 exhaust)	1.5
Total VOC	0	0
	> 0 but < 2	0.5
	≥ 2 but <4	1.0
	≥ 4 but < 6	1.5
	≥ 6	2.0

An example of how a subject's AEI is determined is given below.

Example for Subject Kd20:

First, lets calculate the total VOC for subject Kd20:

VOC (charcoal) = 18.59 ppb; Normalized value = 18.59/73.55 = 0.253
VOC (ORBO) = 1.94 ppb; Normalized value = 1.94/72.84 = 0.027
Average Total VOC (x10) = [(0.253 + 0.027)/2] x 10 = 1.40

Now, lets calculate the scores for each category and the AEI for subject Kd20.

Average Time on Flight-line = 10 h;	score = 1.5
Exposure to Spills = Yes (hydraulic fluid);	score = 0.5
Inhalation Exposure = Yes (JP8 exhaust);	score = 1.5
Total VOC = 1.40;	score = 0.5
Apparent Exposure Index (AEI) =	4.0

The range for AEI scores for all subjects (n = 67) was 0 – 5.5; for all control subjects (n = 20) the range was 0 – 0.5; and for all flight line subjects (n = 47) the range was 1.0 – 5.5. After ranking all flight line subjects, they were divided into three exposure risk categories indicated as low, medium or high (Table 4). Therefore, these exposure risk categories (Low, Medium and High)

were assigned for only flight line subjects. Metadata were not available for three flight line subjects (Kd3, Kd13, and Kd19). Therefore, since their AEI score could not be calculated, these subjects were deleted from analyses where AEIs were used.

Table 4. Subject final AEI ranking for exposure category determination.

AEI Score	Exposure Risk Category	N-Value
4.0 – 5.5	High	16
3.1 – 3.9	Medium	13
1.0 – 3.0	Low	18

4.8 *PCA of U.S. personnel using classification based on NMR spectra and calculated AEI scores*

PCA analysis of all U.S. subjects, minus the two outliers (Kd30 and Yo7), indicated group separation between subjects (Fig. 12A). Interestingly, only the AEI-*high* exposure group (i.e. those subjects who work on the flight line the longest) was found to clearly separate from all other category groups (Fig. 12B). The AEI-*medium* and AEI-*low* groups were both found to overlap with the control group. Another finding with this type of analysis was that the magnitude of the PCA error boundaries increased from AEI-*low* to AEI-*high* subjects. Therefore, it appeared that variability in the urinary metabolite profile increased with increasing exposure risk AEI score.

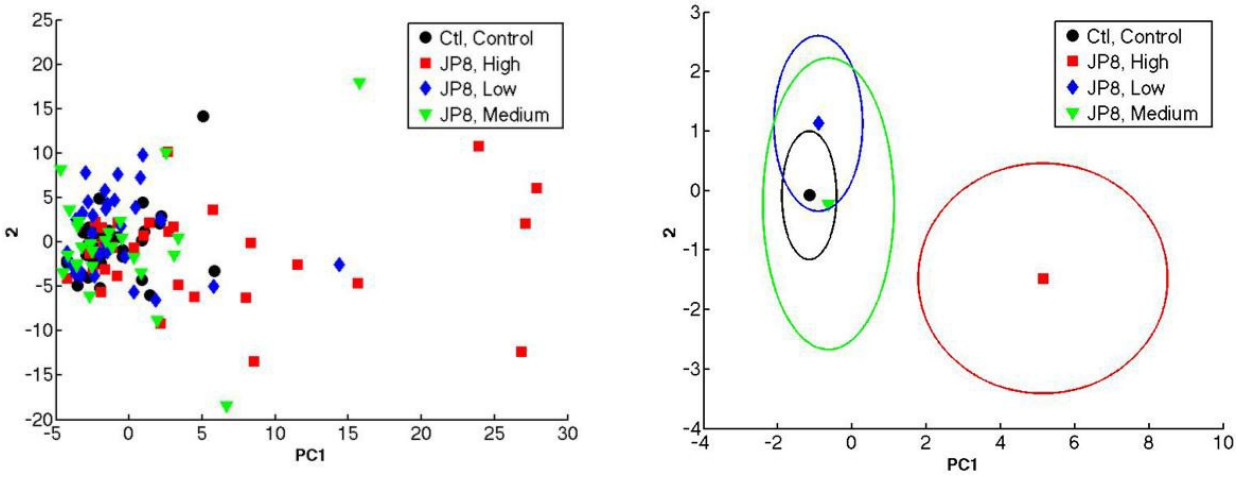

Figure 12. PCA scores plots (PC1 vs. PC2) for control (black) and flight line-exposed U.S. personnel with AEI category of high (red), medium (green) and low (blue) showing all subjects (A) and the centroid Mean ± 2SEM (95% CI; B). Data were autoscaled using the control subjects as reference (both Kd and Yo air bases and pre- + post-shift times). With the two outlier subjects omitted from the analysis, differences between individual subjects were more clearly resolved (A). Additionally, plotting the centroid Mean ± 2SEM (B) shows that the AEI-*high* exposure risk

group clearly separates from all others, while the AEI-*low* and AEI-*medium* exposure groups cluster together with controls.

PCA was then conducted to investigate whether the type of operational aircraft at a particular base (C-130 or F-15) influenced NMR-AEI analysis. Since the AEI-*high* group was the only group clearly separated from control in the above PCA analysis, it was the only group subjected to this type of PCA. A PCA model was utilized that included all control subjects and flight line subjects belonging to the AEI-*high* exposure risk category, that were further grouped by type of aircraft serviced (F-15 or C-130; Fig. 13). All flight line subjects were found to separate from control, but the flight line personnel working at the F-15 air base were found to cluster more tightly (i.e. less variable urinary metabolite profiles) than personnel working at the C-130 air base (Fig.13B). This result indicated that an OPLS-DA should be conducted to examine whether these groups could be classified separately from control subjects with statistical significance, and whether the F-15 flight line personnel yield greater separation with higher significance when compared to personnel working the C-130 flight line.

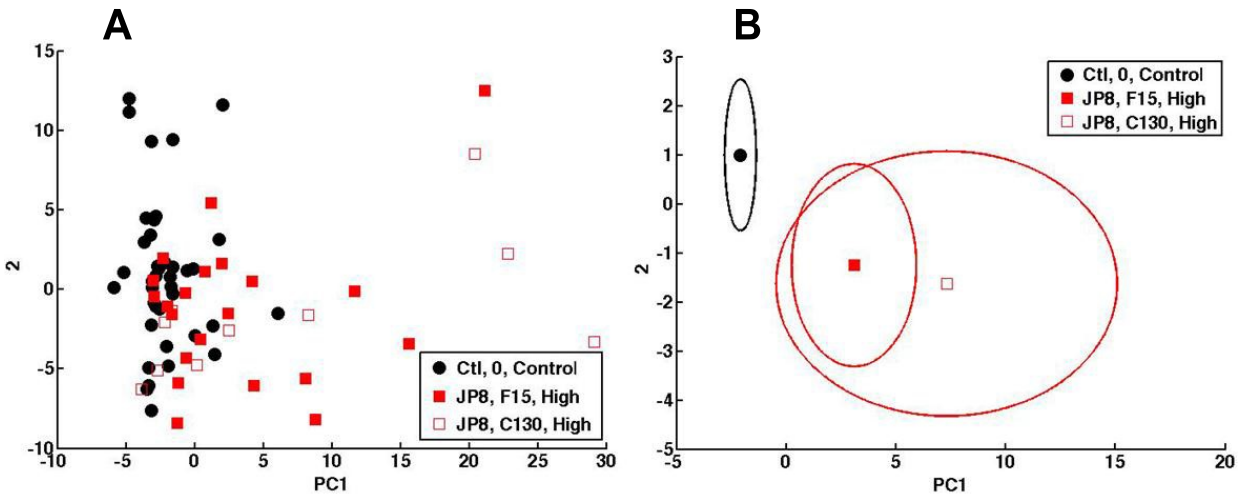

Figure 13. PCA scores plots (PC1 vs. PC2) for control (black) and flight line subjects (red) belonging to the AEI-*High* category with Yo7 and Kd30 omitted. (A) Shows all subjects identifying those working with F-15 (filled squares) and C-130 (open squares) aircrafts, while (B) shows the centroid Mean ± 2SEM (95% CI). Data were autoscaled using the control subjects as reference (both Kd and Yo air bases and pre- + post-shift times).

The OPLS-DA shown in Figures 14 confirms that flight line personnel (AEI-*high*) working at the F-15 air base (Kd) did indeed yield better group separation from control subjects with greater predictive significance (Q^2) than personnel working at the C-130 air base (Yo). Although both OPLS-DAs (minus the two outliers) are significant for both F-15 and C-130 flight line personnel when compared to control ($p < 0.01$), the analysis of the F-15 flight line personnel yielded

greater discriminating power than C-130 flight line personnel (Fig. 14B). Clearly, the T-score plot shows that there was overlap between the control and C-130 flight line personnel (Fig 14A).

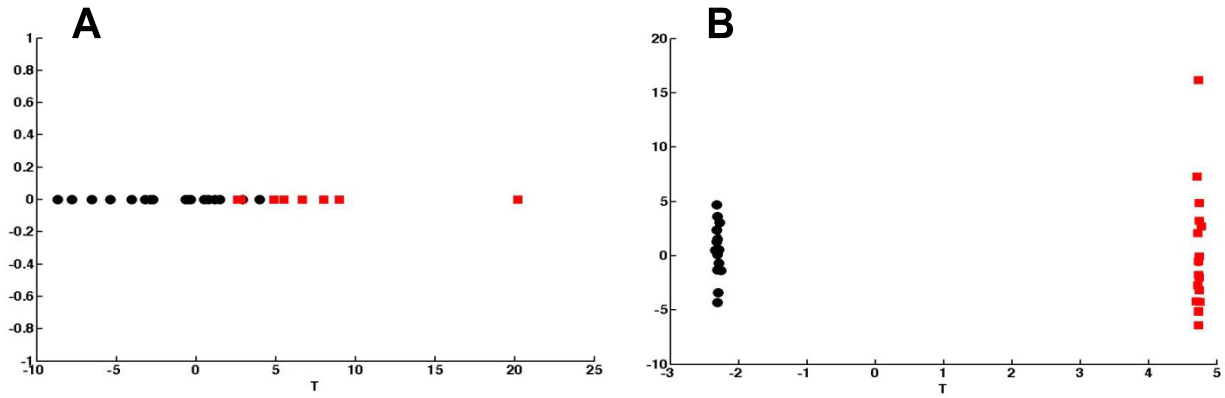

Figure 14. T-score plot derived from OPLS-DA for control (black) *vs.* flight line JP-8 potentially exposed subjects (red) belonging to the AEI-*high* category and working with C-130 (A) or F-15 (B) aircrafts. Subjects Kd30 and Yo7 were excluded in the analyses. Both models are statistically significant ($p < 0.01$). The F-15 air base subjects data (plot B) yielded a greater power of classification with a $Q^2 = 0.637$ (predictive accuracy by leave-on-out cross validation was 100%) compared to C-130 air base subjects data with a $Q^2 = 0.208$. Data were autoscaled using the control subjects as reference (both Kd and Yo airbases and pre- + post-shift times).

Although the predictive significance (Q^2) was increased approximately 2-fold (see Fig. 11) by incorporating AEI metadata into the NMR data analysis of flight line personnel working on JP-8 fueled aircraft (C-130 or F-15) at operational air bases, the predictive power within the flight line personnel group working at the F-15 operational air base (Kd; $Q^2 = 0.637$) was found to be more than three times that observed for flight line personnel working at the C-130 operational airbase (Yo; $Q^2 = 0.208$).

Spectral features identified from the OPLS-DA indicated that 21 significant features (bins or metabolites) were responsible for the differences between control subject urinary metabolite profiles and those of F-15 flight line/AEI-high subject urinary metabolite profiles (Table 5). Interestingly, many of the salient features (bin/metabolites) identified in urine samples from flight line personnel who serviced F-15 aircraft fueled with JP-8 appeared to be in the aromatic region of the NMR spectrum (*ca*. 7-8 ppm), and is in contrast to that observed for the Japanese flight line personnel servicing the same aircraft, but fueled with JP-4 (see Table 2). The OPLS-DA, incorporating AEI-high, for U.S. flight line personnel who serviced C-130 aircraft yielded much less significant discrimination and required a greater number of spectral features (bins) to result in group separation from control subjects (34 bins). However, the most significant feature identified ($\delta = 3.437$ ppm) was identical to that found with flight line personnel who serviced F-15 aircraft (see Table 5). The metabolite signal at 3.44 ppm is a triplet that was identified as

taurine, and its intensity was significantly increased in the flight line personnel who serviced F-15.

Table 5. Listing of significant bins from OPLS-DA for Control *vs.* Flight Line-AEI-*high*-F-15 (subject Kd30 is excluded from this analysis). The chemical shift (δ in ppm) center position is identified for each bin and the data are listed in rank-order from most-to-least significant (highest to lowest |P| value).

Control *vs.* F-15 Flight Line (Q^2 = 0.637; p<0.01)		
δ (ppm)		**|P|**
3.437		0.337128
8.342		0.168497
6.275		0.160080
6.327		0.156927
2.009		0.137889
6.450		0.129063
6.169		0.125935
2.972		0.121388
2.066		0.119224
7.437		0.112553
1.112		0.102458
1.811		0.100827
3.735		0.099507
7.449		0.096974
6.392		0.096416
6.102		0.095621
1.918		0.092675
8.716		0.086476
8.771		0.081840
7.393		0.056712
7.039		0.041162

5.0 DISCUSSION

Previous studies have indicated that naphthalene, 1-naphthol, and 2-naphthol would be good potential biomarkers of exposure to JP-8 (Serdar et al., 2003; Serdar et al., 2004; Smith et al., 2012). However, these studies were conducted using subjects who worked inside fuel tanks where liquid and vapor exposures to JP-8 VOCs would be high. No potential biomarkers have been reported that would be indicative of exposure to JP-4. Since the U.S. military has transitioned from JP-4 to JP-8 use in their aircraft since 1969, it is unlikely that much research has been conducted to monitor for exposure to JP-4.

In the present study, flight line subjects performed a variety of different job-related tasks that included crew chief, weapons, engine and propulsion, fueler, and electrical/environmental. Most of the U.S. flight line personnel involved in the present study were crew chiefs (50%) and would not have had significant exposure to jet fuel directly. This result is further supported by the finding in the present study that no significant difference in 1-naphthol was found in the urine of flight line subjects when compared to control (data not shown).

A review of naphthalene sources indicates that naphthalene is widely distributed pollutant found in ambient and indoor air due to emissions from the chemical and primary metals industries, biomass burning, gasoline and oil combustion, tobacco smoking, the use of mothballs, fumigants and deodorizers, and many other sources (Jia and Batterman, 2010). Furthermore, a recent JP-8 study had shown that a subset of the control subjects exhibited higher constituent levels of JP-8 than the lowest levels found in the exposed group (Pleil et al., 2011). Therefore, unless one is exposed to high levels of JP-8, increase in urinary 1- or 2-naphthol will not be seen as significantly increased over environmental or casual background exposure. Indeed, this has been shown in a recent study of urinary biomarkers of JP-8 among Air Force personnel (Smith et al., 2012). These authors found that job-related predictors of urinary naphthols were among subjects in the high exposure group that included entering fuel tanks, repairing leaks, and direct dermal contact with JP-8. Their results also showed that naphthol levels in the moderate and low exposure groups were similar to the general population, indicating that working on an Air Force base in jobs without high levels of exposure to JP-8 does not necessarily lead to increased naphthol levels compared to the general population. Another previous study of JP-8 exposures at Air Force bases also indicated that the highest overall exposures to JP-8 were experienced by fuel system maintenance workers (Pleil et al., 2000).

Although we could not identify naphthalene or naphthols in the urine of flight line subjects, metabolomics analysis did indicate that these subject's urinary metabolite profiles were significantly different than control subjects and more than likely reflected their exposure to jet fuel combustion by-products. This is partially supported by the finding that urinary profiles determined by NMR analysis among flight line workers servicing different aircraft (F-15 and C-

130) fueled with JP-8 were different (see Fig 11). Greater predictive power for flight line exposure was observed for personnel servicing F-15 aircraft. Since combustion products of JP-8 from F-15 and C-130 engines would be expected to be different, it is possible that this difference in engine combustion is responsible for the observed difference in metabolite profiles of flight line personnel servicing different aircraft with the same fuel. This is further supported by previous studies showing that JP-8 exhaust contains high concentrations of respiratory irritants such as formaldehyde and other non-fully combusted fuel exhaust products (Ritchie et al., 2001; Pleil et al., 2000; Kobayashi and Kikukawa, 2000).

Although our metabolomics results from the present study has yet to identify specific metabolite biomarkers indicative of jet fuel exposure, it was capable of identifying metabolite patterns indicative of flight line exposure. Regardless of fuel (JP-4 or JP-8) it appeared that subjects working with F-15 aircraft have a significantly different urine metabolite profile excreted and here we purport they received a greater flight line exposure than those subjects who worked with C-130 aircraft (see Fig. 6 and 10). After further refinement of the JP-8 dataset, we were able to show distinct metabolite profiles relative to controls for both F-15 and C-130 workers, but again the C-130 data displays greater variability (see Fig. 13). Supervised OPLS-discriminate analyses confirm that flight line subjects working with F-15s, regardless of jet fuel (JP-4 or JP-8), can be classified separately from control subjects with greater discriminating power (Q2 values) and statistical confidence than C-130 flight line workers (see Fig. 8 and 14).

A large variability in urinary metabolite profile was observed for flight line subjects (see Fig. 10C and 10E). There are likely a number of factors that could have been responsible for this that included, but not limited to, job category (i.e. crew chief, weapons, fueler, etc.), time on the flight line, other potential inhalation exposures (i.e. exhaust, gasoline vapors, diesel vapors, etc.) and spill exposures (i.e. hydraulic fluid, solvents, fuels, etc.). In an attempt to factor these additional influences on urinary metabolite profiles, we incorporated metadata collected on each subject into our data processing for NMR data analysis. To accomplish this, we transformed a select number of metadata believed to have an impact on urinary metabolite profiles and developed an “apparent exposure index” (AEI; see Table 3). We were able to group flight line subjects into three AEI categories (low, medium and high). PCA analysis of the NMR data incorporating our transformed metadata (AEI) indicated that only those flight line subjects categorized as AEI-high were found to have urinary metabolite profiles distinct from control subjects (see Fig 12B). Further data analysis by comparing the type of aircraft serviced (F-15 or C-130), now indicated that urinary profiles of flight line personnel servicing either aircraft was distinct from control subjects. However, the urinary profiles of flight line subjects servicing F-15 aircraft showed less variability than urinary profiles from flight line subjects servicing C-130 aircraft (see Fig 13B).

Incorporation of transformed metadata into the multivariate statistical analysis of the NMR data yielded the greatest separation between control subjects and AEI-high flight line subjects servicing F-15 aircraft fueled with JP-8 (Q^2 discriminating power 0.637; predictive accuracy 100%; see Fig 14B). Using this enhanced NMR data analysis method by incorporating metadata for the first time clearly shows that the chosen parameters were found to influence the NMR spectrum. Therefore, we plan to further this type of analysis using the flight line subjects that serviced aircraft fueled with JP-4.

In summary, our metabolomics data suggested that urinary metabolite profiling may be a useful screening tool for monitoring flight line personnel exposures to hazardous volatile chemicals. Furthermore, we have also shown that incorporating select metadata capable of influencing NMR spectral data analysis can enhance the discriminatory power and accuracy of metabolomics analysis. Additional work will be required to identify the key metabolites (biomarkers) predictive of exposure to specific jet fuels or combustion products.

6.0 CONCLUSIONS

A NMR-based metabolomics approach to study flight line personnel exposed to jet fuel directly, and/or to their combustion products, demonstrated observable differences in urinary metabolite profiles that could be distinguished from control air base subjects who were not exposed to local flight line environmental atmosphere. Furthermore, incorporation of collected individual metadata into the metabolomics data analysis scheme was found to increase the OPLS-DA discriminatory power as much as 2-fold for predictive accuracy. An important contributing factor to NMR metabolic profile differences appeared to be related to the type of aircraft being fueled. Personnel working with F-15 aircraft showed greater changes in their excreted urinary metabolite profiles than those individuals working with C-130 aircraft when compared to controls, regardless of air base fuel type (JP-4 or JP-8) in use. Discriminant analysis of multivariate data (OPLS-DA) was able to classify flight line personnel from control subjects with statistical significance ($p<0.01$). The number of significant NMR spectral features (bins) necessary to classify F-15-flightline personnel separately from control subjects was 15 for JP-4 air bases and 21 for JP-8 air bases. These NMR proton bin features appeared to be different for the two fuel types, but this result may be related to the different methods of data analysis used for each dataset (JP-4 air base and JP-8 air base datasets), which highlight different aspects of the dataset. Specifically, NMR analysis of JP-4 air base data used a ‘paired’ approach, that highlighted differences in pre- *vs.* post-shift urine samples from each subject. These data were then used to discern control subjects *vs.* JP-4-flight line personnel. In contrast, the JP-8 air base NMR analyses directly compared flight line-exposed personnel to control subjects without pairing the data. This difference in data processing was imposed as a requirement to account for differences between U.S. and Japanese personnel with regard to scheduling times between working shifts.

"Paired" NMR data analysis would favor greater periods of time between shifts, while direct analysis would favor shorter time periods between working shifts. Further studies are necessary to resolve these issues. The most salient NMR spectral feature, or metabolite, discernible in the JP-8 flight line personnel urinary analysis was an increase found in the excreted levels of taurine. Studies are continuing to identify as many urinary metabolites as possible that lead to group classification, and may provide a set of biomarkers predictive of jet fuel or their combustion products exposure. This study completed phase two of a cooperative research project conducted under a Memorandum of Understanding between the Department of Defense of the United States of America and the Ministry of Defense of Japan. This international agreement, "The Human Effects of Exposure to Aviation Jet Fuels, JP-4 and JP-8, and Their Engine Exhaust," was a scientific collaboration between the 711 HPW/RHDJ (Dr. David Mattie) and JASDF/AML (Dr. Asao Kobayashi). Taken together, our results suggested that urinary metabolite profiling may be useful as a screening tool for monitoring military flight line personnel exposed to hazardous volatile chemicals.

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LIST OF ACRONYMS

AEI	Apparent Exposure Index
AFRL	Air Force Research Laboratory
AML	Aeromedical Laboratory
C-130	Military Transport Aircraft
CI	Confidence Interval
Ctl	Control
D_2O	Deuterium Oxide
F-15	Fighter Aircraft
HPW	Human Performance Wing
JASDF	Japanese Air Self-Defense Force
JP-8	Jet Propulsion fuel 8
JP-4	Jet Propulsion fuel 4
Kd	Kadena Air Base
Ki	Komaki Air base
Ku	Komatsu Air Base
LC/MS	Liquid Chromatography/Mass Spectroscopy
mHz	Megahertz
NATO	North Atlantic Treaty Organization
Nh	Naha Air Base
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
OPLS-DA	Orthogonal Projections onto Latent Structures-Discriminant Analysis
PC	Principal Component
PCA	Principal Component Analysis
ppm	Parts Per Megahertz
Q^2	Coefficient of Prediction
PQN	Probabilistic Quotient Normalization
PRESS	Predicted Residual Sum of Squares
RHDJ	Molecular Bioeffects Branch
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SSY	Sum of Squares “Y”
TSP	Trimethylsilylpropionic (2, 2, 3, 3 d_4) acid
U.S.	United States
USAF	United States Air Force
VOC	Volatile Organic Compound
WPAFB	Wright-Patterson Air Force Base
Yo	Yokoto Air Base

APPENDIX A.

Standard Operating Procedure for Urine Collection

SOP for Urine Collection (Ver 4.0 20 Jul 12)
JP-4 / JP-8 Collaborative Project

1.1 SCOPE AND APPLICATION
1.2 This standard operating procedure describes urine collection for personal in the JP-4 / JP-8 Collaborative Project exposure study.

2.0 EQUIPMENT AND Materials
Scale
Ice in ice bucket or deep tray
Plastic cups (450mL)
Measuring plastic cup (marked in mL)
Screw top amber glass vials (50mL, SU-50A, Nichiden-Rika Glass Co.,Ltd., Tokyo, JP)
Falcon conical tubes (50mL blue/sterile)
Polyethylene storage bags – Ziploc® freezer bags
Sealing film - Labolatry Film (Parafilm®)
Cool boxes - insulated boxes for sample shipping
Cooler gel
AML will purchase above all materials and bring them to Kadena AB and Yokota AB

3.1 Procedure
3.2 Label the empty cup with subjects' code number (YU##, starting with 01 and going sequentially by date)
3.3 Hand two empty cup to subjects
3.4 Measure urine volume in the measuring cup(s) and record the total volume on the coded subject record sheet: Weigh urine cup(s) and record total weight on the coded subject record sheet.
3.5 Place urine into appropriate tubes:
Pour 10 mL into first AFRL tube, cap and put on ice (Metabolomics YUML##)
Pour 10 mL into second AFRL tube, cap and put on ice (Proteomics YUP##)Pour 20 mL into third AFRL tube, cap and put on ice (Metabolomics YUMN##)

Pour 10 mL into Falcon AML tube, cap and put on ice. (Y##1, 2, or 3 for pre, post 1 and post 2)
For AFRL add A for pre-shift; B for postshift and C for next day.
3.6 Seal all tubes and vials with Parafilm
3.7 Keep the Falcon tube samples in Ziploc bags and store at -20 degC.
3.8 Keep the glass vial samples in Ziploc bags and store at 4 degC.

4.1 Instruction for subjects (SOP version)
4.2 Get the empty cup labeled with your code number.
4.3 Have subject take labeled cup to toilet to collect urine.
4.4 Place the cup into the path of the urine stream.
4.5 Start the urine stream and collect in the cup to catch whole portion of your flow.

4.6 Return the collected urine sample to investigator
4.7 Then you can go for blood draw or if blood already drawn and Confidential Information Sheet filled out, you can leave.

4.0 Instruction for subjects (Version given to each subject)

4.1 Get the empty cups labeled with your code number.

4.2 Take labeled cups to toilet to collect urine.

4.3 Place the first cup into the path of the urine stream.

4.4 Start the urine stream and collect in the cup to catch whole portion of your flow until full.

4.5 Switch to second cup to finish flow.

4.6 Return the collected urine sample to investigator.

4.7 Then you can go for blood draw or if blood already drawn and Information Sheet filled out, you can leave.